Bacterial Flora of Seven Species of Fish Collected at Rongelap and Eniwetok Atolls[1]

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A VERY EXTENSIVE LITERATURE exists concerning the normal bacterial flora of marine fish species common to the northern ocean areas, i.e., the North Sea (Stewart, 1932; Aschehoug and Vesterhus, 1943; Reay and Shewan, 1949; Liston, 1956, 1957; Georgala, 1958), the North Atlantic (Reed and Spence, 1929; Gibbons, 1934*a*, 1934*b*; Dyer, 1947), and the North Pacific (Hunter, 1920; Fellers, 1926; Snow and Beard, 1939; Kiser, 1944; Kiser and Beckwith, 1942, 1944; Liston, 1959). These studies of the aerobic heterotrophic bacterial flora found on a number of different species of northern ocean fishes have shown that, while the generic distribution of the bacteria associated with freshly caught marine fish may vary quantitatively, the following genera predominate fairly consistently: *Pseudomonas, Achromobacter, Flavobacterium,* and *Micrococcus.* The genera *Proteus, Sarcina, Bacillus, Corynebacterium,* and *Serratia* are encountered less often. Some investigators have discussed the biochemistry of the organisms isolated from marine fish (viz., Thjøtte and Sømme, 1943) but most of the physiology and biochemistry is limited to only a few properties studied for classifying the microorganisms. A somewhat more extensive discussion of the anabolic and catabolic aspects of the bacterial groups found on North Pacific fish has been given by Colwell (1961) and Liston and Colwell (1962).

There is relatively little information available on the bacteriology of fishes common in warm water areas. Wood (1940, 1950, 1952, 1953) studied marine fish caught off the coast of Australia and reported on the numbers and the types of bacteria found on these animals. Some of his results are at variance with those reported by workers in the northern areas. Thus, he reported large numbers of *Corynebacterium* and *Mycoplana* species from some of the fish sampled. This might indicate that there are differences existing with respect to geographical location of the fish species.

Venkataraman and Sreenivasan (1952, 1954), Velankar (1955, 1956), and Velankar and Kamasastri (1956) carried out bacteriological studies of fish caught in the waters off the coast of India. The results of these workers are at variance with those of Wood, but also indicate peculiar distributions of bacterial genera which suggest that factors related to the physical environment may affect the commensal flora of the fish species.

Periodic studies of the effects of the atomic testing program on the biota of the Marshall Islands have been made since 1946 by the staff of the Applied Fisheries Laboratory, University of Washington (Biddulph and Cory, 1952; Donaldson et al., 1948, 1949, 1956; Palumbo, 1955; Seymour et al., 1957). Since it was relatively simple to arrange for animals collected during these surveys to be sampled bacteriologically, the bacterial flora of the marine fish in southern and northern Pacific Ocean areas could then be compared directly. The bacterial flora of invertebrate animals collected during certain of the atomic testing program studies (Bonham, 1958; Held, 1960) have been analyzed and are reported elsewhere (Colwell and Liston, 1961*a*). The investigations reported in this paper were designed (1) to study the bacterial flora of marine fish of the central Pacific Ocean, i.e., the Rongelap and Eniwetok atolls of the Marshall Islands; (2) to investigate the possibility of geographically

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imposed variation of a natural flora; and (3) to study the question of host specificity in terms of the commensal bacterial flora and the animal host.

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MATERIALS AND METHODS

The animals sampled were the surgeon fish, *Acanthurus triostegus triostegus* (Linnaeus), the snapper, *Aprion virescens* Valenciennes, the jack, *Caranx ferdau* Forskål, the grouper, *Epinephelus merra* Bloch, the goatfish, *Mulloidichthys samoensis* (Gunther), the siganid, *Siganus rostratus* (Valenciennes), and the barracuda, *Sphyraena helleri* Jenkins.

The sampling procedure followed was the sterile-swab technique whereby cotton swabs, sterilized in separate 16 × 150 mm screw-capped culture tubes containing 1–2 cc aged sea water plus 0.5% peptone, were used to sample gill, gut, mouth, and skin areas of the animals. Streaks were then made, in the field, onto the surface of slanted agar in small (1½ oz) prescription bottles. The bottles were transported by air to the College of Fisheries Laboratories at the University of Washington, Seattle, where streakings from the prescription bottles were made onto a medium consisting of 0.5% yeast extract and 0.8% nutrient agar in 1 liter of aged sea water (MacLeod et al., 1954). The colonies appearing after 5 days at 25 C (RT) were picked from the agar into tubes of sea water broth, a Nutrient Broth (Difco) made up with aged sea water in place of distilled water. Each isolate was restreaked three times in order to ensure purity, before the testing program was carried out.

Pure cultures in a medium of 1.0% peptone in sea water were used as the source of organisms for carrying out the descriptive procedures, which tested morphological, physiological, and biochemical characteristics of the bacteria.

Morphology was determined by observation of 24–48 hr cultures, using an American Optical Company "Phasestar" phase-contrast microscope. The Gram stain and the Casares-Gil flagella stain (Manual of Microbiological Methods, 1957) were routinely made. Growth characteristics on agar and in liquid media at 0 C, 25 C (RT) and 37 C were recorded.

Sea water requirement was tested by streak plating on agar containing sea water and on a duplicate distilled water–Nutrient Agar plate. Sensitivity to the antibacterial agents was determined by disc tests (Difco "Uni-discs" and "antibiotic discs") or by ditch tests in the case of the 0/129 compound (Collier, Campbell, and Fitzgerald, 1950; Shewan, Hodgkiss, and Liston, 1954).

Except for the following, all biochemical tests were carried out as described in the Manual of Microbiological Methods (1957): the Hugh and Leifson (1953) test for anaerobic fermentation of sugar was performed; and the trimethylamine oxide (TMO) reduction test as described by Wood and Baird (1943).

For a discussion of the tabulating and handling of the taxonomic data, see Colwell and Liston (1961*b*).

RESULTS AND DISCUSSION

The distribution of bacterial genera within the commensal floras of the seven species of fish sampled in this study is given in Table 1. *Pseudomonas* and *Achromobacter* species were predominant in the floras of *Acanthurus triostegus, Caranx ferdau, Epinephalus merra, Siganus rostrata, and Sphraena helleri. Aprion virescens* yielded more nonfecal enterobacterial species, and *Mulloidichthys samoensis* more of the Gram positive bacterial types. However, the bulk of the flora in six of the seven species of fish studied consisted of the Gram negative, asporogenous, rodlike bacilli of the *Pseudomonas, Vibrio,*

TABLE 1

GENERIC DISTRIBUTION OF AEROBIC HETEROTROPHIC BACTERIA ASSOCIATED WITH SEVEN SPECIES OF MARINE VERTEBRATES

(Expressed as per cent)

ANIMAL SPECIES	*Pseudomonas*	GUT GROUP *Vibrio*	*Achromobacter*	*Flavobacterium*	*Corynebacterium*	*Bacillus*	*Micrococcus*	ENTEROBACTERIA*	OTHER†	TOTAL NUMBER IN SAMPLE
Acanthurus triostegus	36.7	5.3	26.2	10.6	0.0	10.6	0.0	0.0	10.6	19
Aprion virescens	11.1	11.1	22.2	11.1	0.0	0.0	0.0	44.5	0.0	9
Caranx ferdau	44.5	11.1	22.2	0.0	0.0	0.0	11.1	11.1	0.0	9
Epinephalus merra	70.0	10.0	0.0	0.0	0.0	0.0	0.0	20.0	0.0	10
Mulloidichthys samoensis	8.3	4.2	25.0	12.5	12.5	8.3	12.5	0.0	16.7	24
Siganus rostrata	60.0	0.0	0.0	0.0	0.0	20.0	0.0	20.0	0.0	5
Sphraena helleri	0.0	0.0	60.0	20.0	0.0	0.0	20.0	0.0	0.0	5

* *Aerobacter, Aeromonas, Proteus.*
† *Alcaligenes, Brevibacterium, Arthrobacter, Photobacterium,* yeasts.

Achromobacter, and *Flavobacterium* groups. Only one species, the goatfish (*Mulloidichthys samoensis*), showed a balanced flora of Gram positive versus Gram negative types of bacteria. Of the Gram negative types of bacteria forming the commensal floras, *Pseudomonas* and *Achromobacter* species predominated. The Gram positive groups found on the fish varied, i.e., higher numbers of *Bacillus* species were found on *Acanthurus triostegus* and *Siganus rostrata,* and higher numbers of *Micrococcus* species on *Caranx ferdau* and *Sphraena helleri.*

In Table 2, a comparison of the two areas studied is given. *Pseudomonas* species predominated in the Eniwetok samples and *Achromobacter* species in the Rongelap samples.

Studies of bacteria found on animals taken in the waters off the west coast of India and off the coast of Australia (Venkataraman and Sreenivasan, 1952; Wood, 1940) indicated that Gram positive types of bacteria, such as *Micrococcus, Bacillus,* and *Corynebacterium,* tend to predominate significantly in the floras of sea fish from these warm water areas. The results of this study,

TABLE 2

GENERIC DISTRIBUTION OF BACTERIA FOUND ON MARINE ANIMALS

(Expressed as per cent)

AREA OF CAPTURE	*Pseudomonas*	GUT GROUP *Vibrio*	*Achromobacter*	*Flavobacterium*	*Corynebacterium*	*Bacillus*	*Micrococcus*	ENTEROBACTERIA*	OTHER†	TOTAL NUMBER IN SAMPLE
Eniwetok Atoll	52.7	10.6	7.9	2.6	0.0	2.6	2.6	21.0	0.0	38
Rongelap Lagoon	11.1	2.8	27.9	16.7	8.3	11.1	8.3	0.0	13.8	36

* *Aerobacter, Aeromonas, Proteus.*
† *Alcaligenes, Brevibacterium, Arthrobacter, Photobacterium,* yeasts.

as seen from Tables 1 and 2, do not corroborate those findings. Indeed, it would appear that, except for the slightly higher incidence of Gram positive and nonfecal, enterobacterial types, the bacterial flora of the fish of Rongelap and Eniwetok is very similar to that reported for fish in northern waters (Reay and Shewan, 1949; Liston, 1957, 1959; Colwell, 1961).

The great number of *Micrococcus* and *Mycoplana* species reported by Wood (1940, 1953) and of *Bacillus* species reported by Venkataraman and Sreenivasan (1952, 1954) have not been encountered in this study. Since Velankar (1956) and Velankar and Kamasastri (1956) also isolated very high numbers of *Bacillus* species in their studies of fish, it may well be that such factors as fresh water run-off or low sea water-fresh water interchange in the inshore areas where the fish were captured alter the composition of the bacterial commensal floras. Under these circumstances it is not unlikely that *Bacillus* species form a significant part of the commensal bacterial flora of fish of Mandapam and Telicherry, off the west coast of India, as reported by these workers. However, in view of the results obtained in our studies it would be unwise to assume that the associated bacterial flora of fish species inhabiting waters on or near the equator differs significantly from the flora of fish in the northern waters.

In Table 3, the characteristics of bacteria isolated from the seven species of fish are given. Nearly all the cultures tested (76–100%) were capable of growth at 37 C. Thirty to 60% of the cultures peptonized milk, and more than 50% of the cultures liquefied gelatin. However, bacteria isolated from *Aprion virescens* and *Caranx ferdau* were generally less proteolytic, on the basis of the milk peptonization and gelatin liquefaction tests. High urease and nitratase activity was noted in most of the cultures tested. Trimethylamine oxide reducers were isolated from *Acanthurus triostegus, Caranx ferdau,* and *Epinephalus merra.*

Table 4 shows a comparison of carbohydrate degradation tests for bacteria from the seven animal species. As evidenced by the Hugh and Leifson test (1953), 40–78% of all the cultures tested attacked glucose oxidatively and, roughly speaking, 20–67% were glucose fermenting. Cultures taken from *Acanthurus triostegus* were predominantly oxidative in attack on glucose in comparison with the other species tested. Significantly high fermentative attack on carbohydrates, in general, was noted for cultures taken from *Aprion virescens* and *Mulloidichthys samo-*

TABLE 3

BIOCHEMICAL AND PHYSIOLOGICAL CHARACTERISTICS OF BACTERIA ASSOCIATED WITH SEVEN SPECIES OF MARINE VERTEBRATES

(Expressed as per cent positive)

ANIMAL SPECIES	MOTILE	GROWTH AT 0 C	GROWTH AT 37 C	PENICILLIN SENSITIVE	LITMUS MILK PEPTONIZED	GELATIN LIQUEFIED	UREASE POSITIVE	NITRATE REDUCED	TRIMETHYLAMINE OXIDE REDUCED	INDOLE PRODUCED	HYDROGEN SULFIDE PRODUCED	GROWTH IN KOSER'S CITRATE
Acanthurus triostegus	83.4	7.1	76.4	58.9	38.8	83.4	31.2	53.1	25.0	0.0	11.8	35.4
Aprion virescens	55.5	14.3	85.8	22.2	11.1	11.1	28.6	100.0	0.0	0.0	14.3	42.9
Caranx ferdau	88.8	33.3	77.7	33.3	11.1	44.4	25.0	33.3	16.7	0.0	0.0	0.0
Epinephalus merra	100.0	44.4	100.0	0.0	50.0	50.0	44.4	30.0	16.7	0.0	22.2	88.8
Mulloidichthys samoensis	58.3	25.0	100.0	90.0	33.3	70.8	28.2	25.0	0.0	4.2	8.3	12.5
Siganus rostrata	80.0	0.0	100.0	0.0	60.0	100.0	0.0	60.0	0.0	0.0	66.6	0.0
Sphraena helleri	20.0	40.0	100.0	75.0	0.0	60.0	60.0	60.0	*	0.0	0.0	0.0

* Not tested.

TABLE 4

SOME BIOCHEMICAL CHARACTERISTICS, TESTING CARBOHYDRATE UTILIZATION AND DEGRADATION, OF AEROBIC HETEROTROPHIC BACTERIA FROM MARINE FISH

(Expressed as per cent positive)

ANIMAL SPECIES	HUGH AND LEIFSON (Aerobic)	HUGH AND LEIFSON (Anaerobic)	LACTOSE	MALTOSE	MANNITOL	SUCROSE	GALACTOSE	VOGES-PROSKAUER REACTION	METHYL RED REACTION	STARCH HYDROLYZED
Acanthurus triostegus	53.1	5.9	11.8	41.3	29.5	35.4	23.1	0.0	11.8	31.2
Aprion virescens	77.7	66.6	55.5	77.7	71.5	57.2	66.6	28.6	28.6	0.0
Caranx ferdau	44.4	22.2	0.0	11.1	50.0	25.0	16.7	12.5	12.5	16.7
Epinephalus merra	60.0	30.0	33.3	33.3	28.6	22.2	16.7	22.2	0.0	0.0
Mulloidichthys samoensis	54.2	12.5	37.5	58.3	50.0	37.5	45.8	0.0	37.5	8.3
Siganus rostrata	40.0	20.0	33.3	33.3	33.3	33.3	0.0	33.3	66.6	0.0
Sphraena helleri	60.0	20.0	20.0	20.0	20.0	20.0	40.0	0.0	0 0	20.0

ensis. In the overall pattern, for all seven species, relatively high numbers of carbohydrate-utilizing bacteria were isolated from the fish taken in both Rongelap Lagoon and on Eniwetok Atoll.

In their general physiology, therefore, the bacteria from the warm water fish do show differences from the bacteria from cold water fish. The latter have been described as predominantly psychrophilic organisms with a characteristically oxidative metabolism in most cases. This difference may well be related to the higher ambient temperatures of the tropical areas.

SUMMARY

Eighty-one cultures were isolated from seven species of fish captured in Rongelap Lagoon and near Eniwetok Atoll. The composition of the commensal floras, as measured by generic distribution of the bacterial cultures isolated in the samples, showed that the *Pseudomonas/Vibrio, Achromobacter,* and *Flavobacterium* groups predominated. The generic distribution within the floras of the fish captured in the southern areas of the North Pacific Ocean did not indicate that geographical factors effect changes in the commensal floras, insomuch as the data obtained showed good agreement with results obtained by other investigators. Some variations were observed within the bacterial floras of the seven species of fish studied. No species-specific commensal flora was noted.

Biochemical and physiological characteristics studied suggest that the aerobic heterotrophic bacteria commensal to fish inhabiting the waters of Rongelap Lagoon and Eniwetok Atoll are active in proteolysis and in carbohydrate degradation and, in contrast to the psychrophilic bacteria of northern fish, tend to be mesophilic.

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